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FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE **TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371**		ATTORNEY'S DOCKET NUMBER **Beiersdorf 746-KGB** U S APPLICATION NO. (IF KNOWN, SEE 37 CFR 10/019360
INTERNATIONAL APPLICATION NO. **PCT/EP00/03830**	INTERNATIONAL FILING DATE **April 27, 2000 (27.04.00)**	PRIORITY DATE CLAIMED **May 4, 1999 (04.05.99)**

TITLE OF INVENTION

PROCESS FOR THE PREPARATION OF A POLYURETHANE MATRIX WITH COVALENTLY IMMOBILIZED BIOMOLECULES

APPLICANT(S) FOR DO/EO/US

Norbert ETTNER, Thomas MAHLER, Ernst SCHAUMANN, Michael SCHINK

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☐ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

   Copy of first page of published application WO 00/66092

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10/019360	PCT/EP00/03830	Beiersdorf 746-KGB

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS	PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1040.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$890.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$740.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	13 - 20 =	0	x $18.00	**$0.00**	
Independent claims	3 - 3 =	0	x $84.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$890.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$890.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$890.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable)**. ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$890.00**	
				Amount to be: refunded	$
				charged	$

a. ☐ A check in the amount of ________ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **14-1263** in the amount of **$890.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **14-1263** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Kurt G. Briscoe
NORRIS McLAUGHLIN & MARCUS, P.A.
220 East 42nd Street
30th Floor
New York, NY 10017

(212) 808-0700

SIGNATURE

Kurt G. Briscoe
NAME

33,141
REGISTRATION NUMBER

October 25, 2001
DATE

10/019360

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Beiersdorf 746-KGB
6713-Dr. Hn-be

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS	:	NORBERT ETTNER ET AL.
SERIAL NO.	:	TO BE ASSIGNED (THIS APPLICATION IS A 371 OF PCT/EP00/03830 FILED ON APRIL 27, 2000)
FILED	:	HEREWITH
FOR	:	METHOD FOR PRODUCING A POLYURETHANE MATRIX WITH COVALENTLY IMMOBILIZED BIOMOLECULES
ART UNIT	:	UNASSIGNED
EXAMINER	:	UNASSIGNED

October 25, 2001

Hon. Commissioner of Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

SIR:

Prior to examination, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Insert as the first paragraph of the specification the following new paragraph: -- This application is a 371 of PCT/EP00/03830, which was filed on April 27, 2000. Priority of PCT/EP00/03830 is claimed under 35 USC § 120. --

IN THE CLAIMS:

Cancel claim 1 and substitute:

--10. A process for preparing a polyurethane matrix comprising covalently immobilized biomolecules, said process comprising:

a) introducing a polyurethane matrix into a solvent mixture, wherein the solvent mixture comprises at least 80% of at least one nonpolar solvent, the solvent mixture is selected so that the polyurethane matrix swells in the solvent mixture only slightly or not at all, and the solvent mixture contains an activating reagent; and

b) immobilizing biomolecules preferentially on a surface of the polyurethane matrix or on one or more layers of the polyurethane matrix near the surface. --

Amend claims 2-4 to read as follows:

--2. A process as claimed in claim 10, wherein the polyurethane matrix is a polyurethane gel or a polyurethane foam produced therefrom or a polyurethane film produced

therefrom.– –

– –3. A process as claimed in claim 10, wherein the solvent mixture is composed of approximately 80% to 95% of the nonpolar solvents and of approximately 20% to 5% of the polar to slightly polar organic solvents.– –

– –4. A process as claimed in claim 10, wherein the nonpolar solvent is hexane, heptane and/or petroleum ether.– –

Cancel claim 5 and substitute:

– –11. A process for preparing a polyurethane matrix comprising covalently immobilized biomolecules, said process comprising:

a) introducing a polyurethane matrix into water, the polyurethane matrix swelling only slightly or not at all in the water, and the water comprising the activating reagent thiocarbonyldiimidazole; and

b) immobilizing biomolecules preferentially on a surface of the

polyurethane matrix or on one or more layers of the polyurethane matrix near the surface. – –

Amend claim 6 to read as follows:

– –6. A process as claimed in claim 10, wherein the biomolecule is selected from the group consisting of antibodies, chelators, enzyme inhibitors, enzymes, peptides and other proteins.– –

Cancel claim 7 and substitute:

– –12. A method of producing a wound covering, said method comprising the following steps:

a) producing a polyurethane matrix comprising covalently immobilized biomolecules according to the process of claim 10; and

b) forming the polyurethane matrix comprising covalently immobilized biomolecules into a wound covering. – –

Amend claims 8 and 9 to read as follows:

– –8. The method as claimed in claim 7, wherein the biomolecules interact with interfering factors which are present in wound exudate and which interfere with the wound healing process, where the interfering factors are selected from the group consisting of suspended cells and cell fragments, and dissolved constituents, where the interaction comprises a binding, complexation, chelation of the interfering factor or a chemical reaction with the interfering factor, and where the substances are covalently bonded to a carrier material.– –

– –9. The method as claimed in claim 7, wherein the wound covering is selected from the group consisting of dressings, absorbent gauze, bandages, compresses, absorbent coatings, plasters, sheets, films, hydrocolloid dressings, and gels.– –

Please add the following new claims:

– –13. A process as claimed in claim 3, wherein the solvent mixture is composed of 90% of nonpolar solvents and 10% of polar to slightly polar organic solvents. – –

– –14. The method according to claim 8, wherein the dissolved constituents are selected from the group consisting of antigens, free radicals, ions, proteins, peptides, lipids and

[redacted]

free fatty acids. – –

– –15. A wound covering produced by the process of claim 12. – –

– –16. A method of treating a wound comprising applying to a wound a wound covering according to claim 15. – –

REMARKS

The amendments above add a priority claim to the specification, eliminate multiple dependencies to reduce costs, and otherwise place the claims in better form for U.S. substantive examination. Independent claims 1 and 5 are replaced by claims 10 and 11, respectively. Dependent claim 7 is replaced by new claim 12. Amendments have been made to dependent claims 2-4, 6, 8 and 9 to reflect the new dependencies, and also to remove the narrower included ranges in claims 3 and 8, which have been made the subject of new claims 13 and 14, respectively. A clean copy of claims 2-4, 6, 8 and 9 is presented above. A mark-up showing the changes that have been made to these claims using brackets and underlining is attached. Finally, new claims 15 and 16 have been added to cover the wound covering product and the method of using the wound covering product to treat a wound. No new matter has been introduced as these amendments all have clear support in the original specification, and, indeed, follow logically from the original claims.

For the record, Applicants emphasize that although the claims were amended, and, therefore, might be argued to have been amended for a reason substantially related to patentability, a fair reading of the amended claims will reveal that the departures from the previous claims were for clarification purposes only, and that Applicants did not narrow the claims in any material respect. Therefore, Applicants submit that the amended claims are entitled to the full range of equivalents.

Early and favorable action is earnestly solicited.

Respectfully submitted,

NORRIS MCLAUGHLIN & MARCUS, P.A.

By ____________________
Kurt G. Briscoe
Reg. No. 33,141

220 East 42nd Street
30th Floor
New York, New York 10017
Phone: (212) 808-0700
Fax: (212) 808-0700

MARK-UP SHOWING THE CHANGES MADE IN THE PREVIOUS CLAIM TO YIELD THE CLAIM AS AMENDED ABOVE

– –2. A process as claimed in claim [1] 10, wherein the polyurethane matrix is a polyurethane gel or a polyurethane foam produced therefrom or a polyurethane film produced therefrom.– –

– –3. A process as claimed in claim [1] 10, wherein the solvent mixture is composed of approximately 80% to 95% of the nonpolar solvents and of approximately 20% to 5% of the polar to slightly polar organic solvents[, in particular of 90% of the nonpolar solvents and 10% of the polar to slightly polar organic solvents, where the polar to slightly polar organic solvents are selected in particular from the group of acetone, ethers, ketone, esters, admides].– –

– –4. A process as claimed in claim [1] 10, wherein the nonpolar solvent is hexane, heptane and/or petroleum ether.– –

– –6. A process as claimed in [any of the preceding claims] claim 10, wherein the biomolecule is selected from the group consisting of antibodies, chelators, enzyme inhibitors, enzymes, peptides and other proteins.– –

– –8. The [use] method as claimed in [the preceding] claim 7, wherein the biomolecules [covalently bonded in the polyurethane matrix] interact[s] with interfering factors which are present in wound exudate and which interfere with the wound healing process, where the interfering factors are selected from the group consisting of suspended cells and cell fragments, and dissolved constituents [such as antigens, free radicals, ions, proteins, peptides, lipids and free fatty acids], where the interaction comprises a binding, complexation, chelation of the interfering factor or a chemical reaction with the interfering factor, and where the substances are covalently bonded to a carrier material.– –

– –9. The [use] method as claimed in [either of the preceding claims] claim 7, wherein the wound covering is selected from the group consisting of dressings, absorbent gauze, bandages, compresses, absorbent coatings, plasters, sheets, films, hydrocolloid dressings, and gels [and the like].– –

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Beiersdorf Aktiengesellschaft
Hamburg

Description

Process for the preparation of a polyurethane matrix with covalently immobilized biomolecules

The invention relates to a process for the preparation of polyurethane wound coverings on which biomolecules relevant to wound healing, such as, for example, superoxide dismutase, are immobilized, making it possible to initiate and/or promote the normal healing process. A temporary and locally restricted application of the enzymes promoting wound healing compensates for factors which are absent and supplements factors present in insufficient quantity.

Activation of functional groups for covalent coupling of proteins, antibodies and enzymes or low molecular weight organic substances via amino groups is described in detail in standard works such as, for example, W. H. Scouten, Immobilized Enzymes and Cells, in Methods Enzymol., Ed. K. Mosbach, 1987; 135:30 and in Immobilization of Enzymes and Cells, 1997; Ed. G. F. Bickerstaff, Humana Press, Totowa, New Jersey; H. A. Staab, H. Bauer, K. M. Schneider in Azolides in Organic Synthesis and Biochemistry, Wiley-VCH, 1998. Possible functional amino groups for proteins, antibodies and enzymes are the α-amino groups of the amino terminus and the ω-amino groups of the lysine and arginine amino acid side chains exposed on the surface. However, other reagents such as, for example, carbonylditriazole (CDT) (compare C. Delgado et al. 1992, Critical Reviews in Therapeutic Drug Carrier Systems, 9, 249) can also be used for reactivation.

Corresponding coupling processes are known as state of the art and are employed, for example, in the preparation of substances for preparative and analytical applications. Thus, for example, EP 0 087 786 describes a process for immobilizing the iron chelator desferrioxamine via linkage of the primary amino group, with DFO being bonded to agarose-polyaldehyde gel beads. The covalent modification of SOD (P. Pyatak et al., 1980, Res. Com. Chem. Path. and Pharmacol., 29, 115), catalase (A. Abukovski et al., 1976, J. Biol. Chem., 252, 3582) and other enzymes (M. L. Nucci et al., 1991, Advanced Drug Delivery Reviews, 6, 133) with polyethylene

glycol by coupling activated polyethylene glycol onto amino groups of the enzymes is disclosed.

Hirano et al. (1994, J. Controlled Release, 28, 203) describe the preparation of SOD-polymer conjugates with activated divinyl ether and maleic anhydride. Fortier describes in WO 95/15352 the covalent incorporation of peroxidase and catalase via amino groups in a polymer gel consisting of the protein BSA and preactivated polyethylene glycol. Maneke and Polakowski (1981, J. Chrom, 215, 13) describe the immobilization of α-chymotrypsin on a polymer matrix composed of polyvinyl alcohol and terephthalaldehyde.

WO 98/02189 describes a method for coupling enzymes or proteins which are relevant to wound healing to polyhydroxy polymers such as, for example, cellulose. In this case, the swelling of a polyhydroxy polymer in organic solvents is claimed.

Polyurethane gels are likewise polyhydroxy compounds but have the disadvantage, for example in relation to cellulose, that they swell greatly under the conditions known for activation of hydroxyl groups.

In WO 96/31551, proteins or peptides in the dry state are admixed as active agents to polyurethane-crosslinked microgels. The microgels swell in aqueous medium to give hydrogels and release the protein or peptide again from the hydrogel matrix. US 5,000,955 also describes polyurethane hydrogels for cosmetic, biological and medical applications. Cubic phases consisting of glyceryl monooleate are able to immobilize enzymes by noncovalent linkages, as reported in WO 96/39125. In this case, the enzymatic activity is retained through the immobilization and is even increased, compared with the dissolved enzyme, over a lengthy period.

DE 40 26 153 describes the preparation of a polyurethane synthetic foam in whose pores a hydrogel is embedded. Enzymes with proteolytic activity, such as, for example, trypsin, gelatinase, chymotrypsin and collagenase, are bound to the hydrogel, which consists of polysaccharides, via an activation reaction with CDI or the coupling reagent cyanogen bromide. EP 0 236 610 describes the incorporation (encapsulation) of enzymes with oxidative activity, such as, for example, glucose oxidase, into a urethane prepolymer. The enzyme is activated by contact with serum and produces substances with oxidizing activity, such as hydrogen peroxide, for controlling bacteria in infected wounds.

According to DE 36 06 265, therapeutically effective unimmobilized enzymes are incorporated in a polysaccharide-based wound covering for wound cleansing. The proposed enzyme carriers are pieces of cellulose sponge produced by immersion and impregnation in an enzyme solution. After contact of the enzyme carrier with the wound, the unimmobilized enzymes with proteolytic activity are released into the wound.

A proteolytic wound dressing as dry powder or dusting powder in the form of spherical particles of 0.05 to 0.5 mm based on polysaccharides such as dextran, chitin and chitosan, to which a protease is linked, is described in DE 34 44 746. The polysaccharide matrix was activated by use of glutaraldehyde, cyanogen bromide, 2-amino-4,6-dichloro-s-triazine and introduction of isothiocyanate groups with thiophosgene.

The healing of therapy-resistant wounds has since time immemorial been a great challenge to medicine and science. Present-day specifications for the function of interactive coverings for chronic wounds derive from G. Winter (1962, Nature 193, 293) and have recently been reformulated by T. D. Turner (1994, Wound Rep. Reg. 2, 202). The emphasis in this connection is on the creation of a moist wound healing environment which, in contrast to traditional dry wound treatment with, for example, gauze compresses, provides physiological and thus better conditions for the natural processes of wound healing.

The principle of moist wound healing can at present be regarded as the state of the art in the therapy of wounds which heal with difficulty or not at all. The wound covering must absorb most of the exudate but, at the same time, leave on the wound itself a liquid film in which the actual moist wound healing takes place. Dry wounds and those with little exudation must be provided with adequate moisture to achieve rehydration of the dehydrated tissue. In the moist wound which has been established in this way there is then proliferation of new blood vessels and reduced bacterial growth, with a suitable pH being set up. These requirements are met by structures such as, for example, wound coverings which comprise hydrogels, alginates and superabsorbents and which are able to absorb an excess of wound exudate.

The term “interfering factors” means in general materials or substances which impede or retard the wound healing process and thus lead to the development of chronic wounds. These include cells (for example inflammatory cells such as

leukocytes and macrophages) and cell fragments present suspended in the wound exudate, or dissolved constituents such as antigens, free radicals (such as, for example, reactive oxygen species, ROS), ions (such as, for example, iron ions), proteins and peptides.

In the inflammatory phase of wound healing, which follows the blood clotting and blood platelet aggregation after injury and trauma, neutrophils and monocytes preferentially migrate into the damaged tissue. They start there with the phagocytosis of microbes and the breakdown of damaged tissue and foreign antigens. Activation and stimulation by chemical messengers and microorganisms leads to a greatly increased production of ROS, also called the oxidative burst. These ROS are stored in granules and, on further stimulation, released in high local concentrations into the extracellular tissue for controlling microorganisms.

In the normal progress of wound healing, successful elimination of the immunological stimuli is followed by termination of the inflammatory phase, and tissue regeneration can start. However, if these stimuli persist, further leukocytes migrate into the tissue and are in turn activated, leading to a permanently inflamed or chronic wound. The output of ROS (O. Senel et al., 1997, Annals of Plastic Surgery, 39, 516) and proteolytic enzymes leads to tissue damage (Halliwell & Gutteridge, 1989, Free Radicals in Biology and Medicine, 2nd edition, Clarendon Press, Oxford).

It is necessary for the enzyme bound to the polyurethane gels (PU gels) to have a high specificity for the substrate present in the wound fluid. This selective removal or elimination of the substrate improves or initiates the process of healing of chronic wounds, i.e. serious or nonhealing ones.

In the case of an enzyme-doped PU gel comprising the enzymes SOD and/or catalase or a mixture of these enzymes, selective removal of ROS from the wound fluid is possible.
The enzyme SOD catalyzes the dismutation reaction of reactive superoxide to give the less toxic intermediates hydrogen peroxide and oxygen. It occurs as dimeric or tetrameric form. The various enzymes with molecular weights of from 32 000 to 56 000 Da have metal ions such as copper and zinc (Cu-Zn-SOD), manganese (Mn-SOD) or iron (Fe-SOD) as cofactors in the catalytic site. The ubiquitous enzyme SOD plays the major part in the cellular defense against the oxygen-mediated toxicity of ROS and in the regulation of the intracellular oxygen concentration (Fridovich,

1995, Annu. Rev. Biochem., 64, 97). The hydrogen peroxide formed by the SOD reaction is subsequently converted by the redox enzyme catalase, which is ubiquitous in aerobic organisms, in a multistage catalytic cycle into the nontoxic molecules water and oxygen (Gouet et al., 1996, Nature Structural Biology, 3, 951). Apart from catalase, enzymes also capable of degrading hydrogen peroxide are glutathione peroxidase and myeloperoxidase.

It is an object of the invention to produce by a novel process a water-insoluble, water-absorbing polyurethane matrix which can be employed for medical purposes and is particularly suitable for promoting the healing of chronic wounds.

The object is achieved according to the invention by immobilizing enzymes which interact with the ROS present in the wound fluids of chronic wounds, i.e. with factors which impede the wound healing process, during preparation of the polyurethane matrix, where these additional substances are covalently bonded to the PU matrix.

Accordingly, the invention describes a process for the preparation of a polyurethane matrix with covalently immobilized biomolecules, where the polyurethane matrix is introduced into a solvent mixture, where the solvent mixture comprises at least 80% nonpolar solvents (in particular between 80% and 95%), in which the polyurethane matrix swells only slightly or not at all and in which the activating reagent is present, so that the subsequent immobilization of the biomolecule takes place preferentially on the surface of the polyurethane matrix or on layers near the surface.

In a first preferred embodiment, the polyurethane matrix is a polyurethane gel or a polyurethane foam produced therefrom or a polyurethane film produced therefrom.
The essentially anhydrous and in some cases self-adhesive PU gel compositions are disclosed, for example, in DE 196 18 825, EP 0 057 839, EP 0 147 588 and DE 43 08 347. The gels described therein are composed of polyhydroxy compounds and aromatic or aliphatic polyisocyanates. Preference is given to the use of a polyurethane gel composed of Levagel (copolymer of propylene oxide and ethylene oxide with a molecular weight of about 6 400 g/mol, Bayer, Leverkusen) and hexamethylene diisocyanate with an NCO/OH ratio of from 0.3 to 0.7. Since not all OH functionalities react during the crosslinking reaction, free OH functionalities are available for covalent coupling to a PU matrix for immobilization of substances in the manner described herein.
It is advantageous for use of the polyurethane products of the invention as wound

covering if the immobilized substances are connected directly to the PU surface. Solvents which dissolve the activating reagent are necessary for the activation reaction. The polyurethane swells so much in acetone, dioxane, diethyl ether, dimethyl glycol or dichloromethane, and alcohols and in mixtures of said solvents, are that it tears even under slight mechanical stress. In addition, low molecular weight constituents of the polyurethane gel are dissolved out of the gel matrix. This results, on the one hand, in loss of precisely the constituents having many free OH groups for activation and coupling and, on the other hand, in changes in the mechanical properties of the polyurethane gel. In addition, if the PU matrix swells greatly the substances may also be partly immobilized inside the PU matrix and thus are useless for the application.

The solvent mixture comprises in particular approximately 80% to 95% of nonpolar solvents and approximately 20% to 5% of the polar to slightly polar organic solvents, in particular 90% of the nonpolar solvents and 10% of the polar to slightly polar organic solvents.

The polar to slightly polar organic solvents are, in particular, chosen from the group of acetone, ethers, ketones, esters, amides, for example methyl tert-butyl ketone, dioxane, diethyl ether, dimethylglycol, dichloromethane, ethyl acetate, dimethylformamide. The nonpolar solvents preferably employed are hexane, heptane and/or petroleum ether (for example petroleum ether 35/60).

The polyurethane matrix scarcely swells in nonpolar solvents such as hexane, heptane or petroleum ether. The activating reagents such as, for example, CDI or CDT are virtually insoluble in these solvents. On use of a solvent mixture composed of 80% to 95% of the nonpolar solvents such as hexane, heptane or petroleum ether, and 20% to 5% of the polar to slightly polar organic solvents, the swelling and thus the disadvantages mentioned are greatly reduced but, nevertheless, sufficient activating reagent can dissolve.

The polyurethane gel likewise shows only a slight tendency to swell in water, although the use of water in general as solvent for the activation is precluded because CDI and CDT decompose in water. An alternative activating reagent which can be employed to solve this problem is thiocarbonyldiimidazole (TCDI).

In this alternative possible achievement, a process for the preparation of a polyurethane matrix with covalently immobilized biomolecules is claimed, where the polyurethane matrix is introduced into water as solvent for the TCDI (about 1% by weight), in which the polyurethane matrix swells only slightly or not at all, and in

which the activating reagent is present, so that the subsequent immobilization of the biomolecule preferentially takes place on the surface of the polyurethane matrix or on layers near the surface.

In a further preferred variant, the biomolecule is selected from the group consisting of antibodies, chelators, enzyme inhibitors, enzymes, peptides and other proteins.

The polyurethane matrix prepared according to the invention can be employed particularly advantageously for producing wound coverings. The biomolecule covalently bonded to the polyurethane matrix ought to interact with interfering factors which are present in the wound exudate and which impede the wound healing process, where the interfering factors are selected from the group consisting of suspended cells and cell fragments, and dissolved constituents such as antigens, free radicals, ions, proteins, peptides, lipids and free fatty acids, where the interaction comprises a binding, complexation, chelation of the interfering factor or a chemical reaction with the interfering factor, and where the substances are covalently bonded to a carrier material.
It is possible, for example, with the enzyme-immobilized polyurethane gels to render toxic ROS in the wound fluid of chronic wounds harmless.

The wound covering is selected in particular from the group consisting of dressings, absorbent gauze, bandages, compresses, absorbent cottons, plasters, sheets, films, hydrocolloid dressings, gels and the like.

Finally, the wound covering ought to be able to absorb moisture.

It is preferred for the interfering factors to be iron ions and for the substance interacting with the interfering factors to be a chelator, the latter in turn being selected in particular from the group of immobilizable complexing agents such as, for example, desferroxamine.

It is further preferred for the interfering factors to be reactive oxygen free radicals and for the substance interacting with the interfering factors to be a free radical trap, the latter being selected in particular from the group consisting of superoxide dismutase, catalase, glutathione peroxidase, myeloperoxidase and enzyme mimics or another combination thereof.

It is further preferred for the interfering factor to be a protease and for the substance interacting with the interfering factor to be a protease inhibitor, the latter being selected in particular from the group consisting of natural proteinogenous protease inhibitors from the class of tissue inhibitors of matrix metalloproteinases such as, for example, alpha-2 antiplasmin, alpha-2 macroglobulin, alpha-1 antichymotrypsin, soybean trypsin inhibitor and alpha-1 protease inhibitor.

It is then possible for the interfering factors to be iron ions and for the substance interacting with the interfering factors to be desferrioxamine, or reactive oxygen free radicals and for the substance interacting with the interfering factors to be a free radical trap, the free radical trap being selected from the group consisting of superoxide dismutase, catalase, glutathione peroxidase, myeloperoxidase and enzyme mimics or a combination thereof.

A further possibility is for the interfering factor to be a protease and for the substance interacting with the interfering factor to be a protease inhibitor, the protease inhibitor being selected from the group consisting of natural proteinogenous protease inhibitors from the class of tissue inhibitors of matrix metalloproteinases, aprotinin, alpha-2 antiplasmin, alpha-2 macroglobulin, alpha-1 antichymotrypsin, soybean trypsin inhibitor and alpha-1 protease inhibitor.

The invention further describes the possibility of coupling also via spacer molecules, that is to say α,ω-difunctional substances such as, for example, 1,6-diaminohexane or 6-aminohexanecarboxylic acid, to a PU matrix.

The present invention makes available for the first time PU gel matrices prepared by a novel process as wound covering for improving the process of healing of chronic wounds.

When the PU matrix is removed, the active biomolecules are likewise removed without remaining in the wound or in the wound fluid. The biomolecules which are used for the interaction and are covalently embedded in the PU matrix are thus introduced only temporarily into the wound and are removed again from the wound region after they have carried out their task as intended (i.e. engaged in the abovementioned interactions). The selective removal or elimination of the toxic ROS, the proteolytic decomposition of dead tissue and the killing of microorganisms improves or initiates the process of healing of chronic wounds, i.e. serious or

nonhealing ones.

There is a distinctly increased protease activity in the chronic wound (Weckroth et al.,1996, J. Invest. Dermatol., 106, 1119; Grinell & Zhu, 1996, J. Invest. Dermatol. 106, 335) compared with the acute wound. Enzymes such as, for example, SOD and catalase which, owing to their protective effect, act to promote wound healing would be proteolytically degraded thereby and thus become inactive. A polyurethane gel to which such enzymes are covalently bonded acts as a shield and can prevent the attack of proteases, as has been shown with polyethylene glycol-modified dissolved enzymes (J. S. Beckman et al., 1988, J. Biol. Chem., 263, 6884; Y. Inada et al., 1995, Tibtech, 13, 86).

It is thus possible within the framework of the present invention not only to generate a moist wound environment in order to improve the process of healing of chronic wounds, but also thereby to expedite further according to the invention the healing process, for example through the abovementioned transformation processes.

The present invention is explained below by means of examples without intending unnecessarily to restrict it thereby.

Examples

The polyurethane gels employed for the following examples comprised a polyol component (Levagel, Bayer, Leverkusen) and a diisocyanate component such as, for example, hexamethylene diisocyanate (Bayer, Leverkusen) with an NCO/OH ratio of from 0.3 to 0.7.

Example 1
Preparation of a polyurethane matrix activated with N,N'-carbonyldiimidazole (CDI) or N,N'-carbonylditriazole (CDT)

The experiments on the preparation of a CDI-activated or CDT-activated polyurethane matrix were carried out as follows.
10 identical pieces of polyurethane (thickness 1.2 mm, diameter 15 mm, 275 mg/piece) were activated in a mixture of 90 ml of dry hexane (or petroleum ether 35/60) and 10 ml of dry acetone with 1 g (6.25 mmol) of CDI (Sigma, Steinheim) or

1 g (6.1 mmol) of CDT (Sigma, Steinheim).

In both cases, the reaction in a 250 ml round-bottomed flask took place at room temperature with exclusion of moisture and moderate stirring for one hour. The solvent was then decanted off, and the CDI-activated or CDT-activated pieces of polyurethane were dried to constant weight on siliconized release paper in the air.

Example 2

Preparation of a polyurethane matrix activated with thiocarbonyldiimidazole (TCDI)

10 identical pieces of polyurethane were activated as described in Example 1 with 1 g of thiocarbonyldiimidazole (TCDI, Fluka, 5.6 mmol) in 100 ml of distilled water. The reaction took place at 32°C with moderate stirring in a 250 ml round-bottomed flask for two hours.

The water was then decanted off, and the pieces of PU were washed three times with distilled water and then dried on siliconized release paper.

Example 3

Coupling of the spacer molecules 6-aminohexanoic acid or 1,6-diaminohexane to a polyurethane matrix

10 identical pieces of polyurethane were activated as described in Example 1 and then placed in 20 ml of $NaHCO_3$ buffer (100 mM, pH 8.0), referred to hereinafter as coupling buffer. To this were added either 500 mg (3.81 mmol) of 6-aminohexanoic acid, analytical grade (Serva, Heidelberg) or 500 mg (4.3 mmol) of 1,6-diaminohexane (Fluka, Buchs).

The reaction took place at room temperature with vigorous stirring for 18 hours. The buffer solution was then poured off. The pieces of polyurethane were then washed a total of three times each with distilled water in order to remove residues of uncoupled 6-aminohexanoic acid or 1,6-diaminohexane. The pieces of polyurethane coupled with 6-aminohexanoic acid or 1,6-diaminohexane were then dried to constant weight in the air.

Example 4
Coupling of deferoxamine (DFO) to an activated polyurethane matrix

10 identical pieces of polyurethane activated as stated in Examples 1 or 2 were placed in 20 ml of coupling buffer. Then 200 mg of DFO (0.30 mmol) (Sigma, Deisenhofen) were added in each case. The coupling reaction took place with vigorous stirring at room temperature for 18 hours. The buffer solution was then poured off and replaced by distilled water several times in order to wash out nonspecifically bound DFO from the polymer.

The product of the polyurethane and DFO coupling was detected by a color reaction which also represents a functionality assay: complexation of iron with dissolved DFO results in the ratio 1:1 DFO-iron(III) complex ferrioxamine which has an intense orange color (Hallaway et al., 1989, PNAS, 86, 10108). The DFO-coupled polyurethane matrix was incubated with 200 μM iron sulfate (pH 5.0) with gentle stirring for 2-3 hours, the polyurethane matrix becoming brownish red in color, proving the immobilization of DFO. Finally, the polyurethane matrix was washed several times with distilled water in order to remove unbound iron. The color of the samples intensified after leaving to stand overnight. A check with unactivated polyurethane led to no coloration with iron sulfate solution.

Example 5
Coupling of bovine serum albumin (BSA) to an activated polyurethane matrix

10 identical pieces of polyurethane were activated as described in Example 1 and then placed in 20 ml of coupling buffer. To this were added 200 mg of BSA (Serva, receptor grade, lyophilized, 2.985 μmol).

The reaction took place at room temperature while stirring vigorously for 18 hours. The buffer solution was then decanted off, and the pieces of polyurethane were washed a total of three times in order to detach nonspecifically bound protein from the polyurethane matrix, first with a 0.1% strength aqueous polyoxyethylene sorbitan monolaurate solution (Tween-20) from Fluka (Buchs), then with saturated NaCl solution (Merck, Darmstadt) and lastly with distilled water.

To detect the BSA immobilized on polyurethane, a staining was carried out with the fluorescent dye fluorescamine (Fluram®) from Fluka (Buchs).

15 mg of the dye were dissolved in 10 ml of dry acetone. 250 µl of the fluorescamine solution were added to a piece of polyurethane coupled to BSA, which was placed in 1 ml of an $NaHCO_3$ buffer (100 mM, pH = 8.0). The fluorescence was observed with a Fluovert FS (Leica, Bensheim) fluorescence microscope at an excitation frequency of 390 nm and an emission frequency of 475 nm.

The dye reacted specifically with primary amino groups in the protein (S. Udenfried et al., 1972, Science 178, 871), the hydrolysis products and the dye themselves not being fluorescent. Decomposition of the dye and formation of the fluorescent product took place very quickly in aqueous solution so that no interference by side reactions occurred.

Proteins immobilized on the polyurethane matrix can be stained with trinitrobenzene-sulfonic acid (P. Cuatrecasas, C. B. Anfinsen, Affinity Chromatography, Methods of Enzymology, 1971, 22, p. 363). 500 µl of a 5% strength aqueous TNBS solution were added to a piece of polyurethane with immobilized BSA in 30 ml of distilled water. This solution was heated on a water bath with stirring for one hour. Nonspecifically bound dye was then removed from the polyurethane matrix with distilled water. This process was repeated until the washing water remained colorless. It was possible to show that the enzyme-coupled polyurethane matrix exhibited by comparison with an untreated polyurethane matrix an orange color of the polyurethane matrix.

Example 6
Coupling of superoxide dismutase (SOD) to an activated polyurethane matrix

The enzyme employed was yeast SOD, Dismutin® BT (Pentapharm, Basle). Before being used in the coupling reaction with the activated polyurethane matrix, the buffer in the SOD solution was changed in order to remove stabilizers such as, for example, parabens. This was done by pipetting 2 ml of the SOD solution into a micro-concentrator (Amincon, Witten) with an exclusion volume of 10 kDa. This was followed by centrifugation in a centrifuge (Hettich, Model EBA 3S, Tuttlingen) at a speed of 3 000 rpm for 30 minutes. The filtrate was discarded and the supernatant was made up with 1 ml of the coupling buffer or water. This procedure was repeated

3 times, and then the SOD solution was employed in a concentration of 1.5 mg/ml (1:10 dilution) for the coupling with the activated polyurethane.

The coupling reaction was carried out with 10 identical pieces of polyurethane using the activation reactions from Example 1. The coupling conditions and workup of the products are identical to those described in Example 5 for the coupling of BSA to a polyurethane matrix.

Detection of the coupling of the yeast SOD to the polyurethane matrix was carried out by an ELISA. Firstly the SOD-immobilized PU gel matrix was incubated with a human anti-sheep (C7/Zn) superoxide dismutase antibody (Calbiochem, No. 574597) in a dilution of 1:500 at room temperature for one hour in a 24-well cell culture plate (Greiner, Frickenhausen). A secondary antibody conjugated to a peroxidase bound to this primary anti-(Cu/Zn) SOD antibody. The substrate tetra-methylbenzidine (TMB) was converted by the peroxidase, resulting in a color reaction which can be quantified by spectroscopy. After the color reaction was stopped, 150 μl portions of the solutions were transferred into a well of a 96-well microtiter plate and determined by photometry at 450 nm in an ELISA reader MR 5000 (Dynex, Denkendorf) with a 550 nm reference.

For the positive control, a 96-well microtiter plate with round bases (Greiner, Frickenhausen) was coated with 20 μg/ml yeast SOD Dismutin® BT (Pentapharm, Basle) in 1 ml of buffer (50 mM $NaHCO_3$, pH 9.4) per well and incubated in a refrigerator at 4°C overnight. Then 1 ml portions of 20% strength porcine serum (Gibco, Karlsruhe) were used per well to block nonspecific binding on a shaker at room temperature for one hour. After washing 4 times with PBS buffer (135 mM NaCl, 3 mM KCl, 8 mM KH_2PO_4, 1.5 mM Na_2HPO_4, pH 7.5) with 0.01% Tween-20 (Merck, Darmstadt), the primary anti-sheep (Cu/Zn) superoxide dismutase antibody was incubated on the shaker in a dilution of 1:500 in dilution buffer (0.5% Tween-20, 2% porcine serum in PBS buffer) with 1 ml in each well at room temperature for one hour. This was followed by a further 4 washes with washing buffer, and peroxidase-conjugated anti-sheep IgG (Dianova, No. 713-035-147) was incubated as secondary antibody in a dilution of 1:10 000 with 1 ml in each well on a shaker at room temperature for one hour. After a further 4 washes with washing buffer, the TMB substrate solution was prepared by dissolving (10 mg/ml DMSO) and subsequently adding 70 μl of TMB stock solution with 10 ml of acetate buffer. 1 ml of the TMB solution was added to each well and, after starting with 13 μl of H_2O_2 (3%),

incubation on a shaker was carried out at room temperature for 20 min. After 20 min, the resulting color reaction was stopped by adding 140 µl of 2M H_2SO_4 per well, and photometric determination at 450 nm was carried out in an ELISA reader MR 5000 with a 550 nm reference.

Example 7
Coupling of yeast SOD to a polyurethane matrix via the spacers 6-aminohexanoic acid and 1,6-diaminohexane

Pieces of polyurethane pretreated with a 6-aminohexanoic acid or 1,6-diaminohexane spacer as described in Example 2 were placed in 20 ml of distilled water (pH adjusted to 4.5 with 0.02 M hydrochloric acid). While stirring gently, 500 mg (2.61 mmol) of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were added thereto and, after 1 minute, 10 ml of an SOD solution (1.5 mg/ml) were added. The coupling reaction of the yeast SOD onto the polyurethane matrix took place with gentle stirring at room temperature for 18 hours. The procedure for work-up and purification of the polymer was as described for the coupling of bovine serum albumin (BSA) to an activated polyurethane matrix (Example 5). An ELISA was carried out to detect the coupling reaction (see Example 6).

Patent claims

1. A process for the preparation of a polyurethane matrix with covalently immobilized biomolecules, where the polyurethane matrix is introduced into a solvent mixture, where the solvent mixture comprises at least 80% nonpolar solvents, in which the polyurethane matrix swells only slightly or not at all and in which the activating reagent is present, so that the subsequent immobilization of the biomolecule takes place preferentially on the surface of the polyurethane matrix or on layers near the surface.

2. A process as claimed in claim 1, wherein the polyurethane matrix is a polyurethane gel or a polyurethane foam produced therefrom or a polyurethane film produced therefrom.

3. A process as claimed in claim 1, wherein the solvent mixture is composed of approximately 80% to 95% of the nonpolar solvents and of approximately 20% to 5% of the polar to slightly polar organic solvents, in particular of 90% of the nonpolar solvents and 10% of the polar to slightly polar organic solvents, where the polar to slightly polar organic solvents are selected in particular from the group of acetone, ethers, ketones, esters, amides.

4. A process as claimed in claim 1, wherein the nonpolar solvent is hexane, heptane and/or petroleum ether.

5. A process for the preparation of a polyurethane matrix with covalently immobilized biomolecules, where the polyurethane matrix is introduced into water as solvent in which the polyurethane matrix swells only slightly or not at all and in which the activating reagent thiocarbonyldiimidazole is present, so that the subsequent immobilization of the biomolecule takes place preferentially on the surface of the polyurethane matrix or on layers near the surface.

6. A process as claimed in any of the preceding claims, wherein the biomolecule is selected from the group consisting of antibodies, chelators, enzyme inhibitors, enzymes, peptides and other proteins.

7. The use of the polyurethane matrix prepared as claimed in any of the preceding claims for producing wound coverings.

8. The use as claimed in the preceding claim, wherein the biomolecule covalently bonded in the polyurethane matrix interacts with interfering factors which are present in wound exudate and which interfere with the wound healing process, where the interfering factors are selected from the group consisting of suspended cells and cell fragments, and dissolved constituents such as antigens, free radicals, ions, proteins, peptides, lipids and free fatty acids, where the interaction comprises a binding, complexation, chelation of the interfering factor or a chemical reaction with the interfering factor, and where the substances are covalently bonded to a carrier material.

9. The use as claimed in either of the preceding claims, wherein the wound covering is selected from the group consisting of dressings, absorbent gauze, bandages, compresses, absorbent coatings, plasters, sheets, films, hydrocolloid dressings, gels and the like.

Abstract

A process for the preparation of a polyurethane matrix with covalently immobilized biomolecules, where the polyurethane matrix is introduced into a solvent mixture, where the solvent mixture comprises at least 80% nonpolar solvents, in which the polyurethane matrix swells only slightly or not at all and in which the activating reagent is present, so that the subsequent immobilization of the biomolecule takes place preferentially on the surface of the polyurethane matrix or on layers near the surface.

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ATTORNEY DOCKET NO.: Beiersdorf 746-KGB
6713-Dr. Hn-be

COMBINATION DECLARATION & POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROCESS FOR THE PREPARATION OF A POLYURETHANE MATRIX WITH COVALENTLY IMMOBILIZED BIOMOLECULES

the specification of which was filed on October 25, 2001

as U.S. Serial No. 10/019,360, which is a 371 of PCT/EP00/03830 and

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

(Number)	(Country)	(Day/Month/Yr. Filed)	Priority Claimed
199 20 262.1	Germany	4 May 1999	X yes ___ no
			___ yes ___ no

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented,pending,abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punished by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Kurt G. Briscoe, Reg. No. 33,141; William C. Gerstenzang, Reg. No. 27,552; Lorimer P. Brooks, Reg. No. 15,155; Bruce Londa, Reg. No. 33,531; Christa Hildebrand, Reg. No. 34,953; Howard C. Lee, Reg. No. 48,104; and Theodore A. Gottlieb, Reg. No. 42,597 all of 220 East 42nd Street, 30th Floor, New York, New York 10017; William R. Robinson, Reg. No. 27,224; Davy E. Zoneraich, Reg. No. 37,267, Mark A. Montana, Reg. No. 44,948 and Robert A. Hyde, Reg. No. 46,354, of 721 Route 202-206, Bridgewater, New Jersey 08807, my attorneys and/or agents with full power of substitution and revocation.

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SEND CORRESPONDENCE TO:
NORRIS, McLAUGHLIN & MARCUS
220 EAST 42ND STREET - 30TH FLOOR
NEW YORK, NEW YORK 10017

DIRECT TELEPHONE CALLS TO:
KURT G. BRISCOE
(212) 808-0700

1-OD

FULL NAME OF SOLE OR FIRST INVENTOR: **NORBERT ETTNER**	
INVENTOR'S SIGNATURE: Norbert Ettner	DATE: 15.05.2002
RESIDENCE: Ludwig-Thoma-Strasse 21, D-86551, Aichach, Germany DEX	CITIZENSHIP: German

2-OD

FULL NAME OF SECOND INVENTOR: **THOMAS MAHLER**	
INVENTOR'S SIGNATURE: Th. Mahler	DATE: 30.05.2002
RESIDENCE: Rollplatz 3B, D-38678 Clausthal-Zellerfeld, Germany DEX	CITIZENSHIP: German

3-OD

FULL NAME OF THIRD INVENTOR: **ERNST SCHAUMANN**	
INVENTOR'S SIGNATURE: Ernst Schaumann	DATE: 30.5.2002
RESIDENCE: Kurt-Küchler-Strasse 39, D-22609 Hamburg, Germany DEX	CITIZENSHIP: German

4-OD

FULL NAME OF FOURTH INVENTOR: **MICHAEL SCHINK**	
INVENTOR'S SIGNATURE: Michael Schink	DATE: 03.04.2002
RESIDENCE: Falckweg 3, D-22605 Hamburg, Germany DEX	CITIZENSHIP: German

FULL NAME OF FIFTH INVENTOR:	
INVENTOR'S SIGNATURE:	DATE:
RESIDENCE:	CITIZENSHIP:

FULL NAME OF SIXTH INVENTOR:	
INVENTOR'S SIGNATURE:	DATE:
RESIDENCE:	CITIZENSHIP:

FULL NAME OF SEVENTH INVENTOR:	
INVENTOR'S SIGNATURE:	DATE:
RESIDENCE:	CITIZENSHIP: